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## **Bartonella henselae engages inside-out and outside-in signaling by integrin 1 and talin1 during invasome-mediated bacterial uptake**

Truttmann, M C ; Misselwitz, B ; Huser, S ; Hardt, W-D ; Critchley, D R ; Dehio, C

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# *Bartonella henselae* engages inside-out and outside-in signaling by integrin $\beta 1$ and talin1 during invasome-mediated bacterial uptake

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## Summary

The VirB/D4 type IV secretion system (T4SS) of the bacterial pathogen *Bartonella henselae* (*Bhe*) translocates seven effector proteins (BepA–BepG) into human cells that subvert host cellular functions. Two redundant pathways dependent on BepG or the combination of BepC and BepF trigger the formation of a bacterial uptake structure termed the invasome. Invasome formation is a multi-step process consisting of bacterial adherence, effector translocation, aggregation of bacteria on the cell surface and engulfment, and eventually, complete internalization of the bacterial aggregate occurs in an F-actin-dependent manner. In the present study, we show that *Bhe*-triggered invasome formation depends on integrin- $\beta 1$ -mediated signaling cascades that enable assembly of the F-actin invasome structure. We demonstrate that *Bhe* interacts with integrin  $\beta 1$  in a fibronectin- and VirB/D4 T4SS-independent manner and that activated integrin  $\beta 1$  is essential for both effector translocation and the actin rearrangements leading to invasome formation. Furthermore, we show that talin1, but not talin2, is required for inside-out activation of integrin  $\beta 1$  during invasome formation. Finally, integrin- $\beta 1$ -mediated outside-in signaling by FAK, Src, paxillin and vinculin is necessary for invasome formation. This is the first example of a bacterial entry process that fully exploits the bi-directional signaling capacity of integrin receptors in a talin1-specific manner.

**Key words:** *Bartonella henselae*, Type IV secretion system, Integrins, Invasome formation, Talins

## Introduction

The zoonotic pathogen *Bartonella henselae* (*Bhe*) causes persistent asymptomatic infections in cats that serve as a mammalian reservoir host. Accidental transmission of *Bhe* to humans by cat scratches or bites can manifest in a broad spectrum of clinical symptoms. Although immunocompetent patients typically develop cat-scratch disease (Florin et al., 2008), immunocompromised patients often suffer from bacillary angiomatosis or peliosis, which is characterized by vaso-proliferative lesions in liver or skin, respectively (Dehio, 2005).

*Bhe* expresses a VirB/D4 type IV secretion system (T4SS), which mediates the translocation of seven *Bartonella* effector proteins, BepA to BepG, into host cells (Schmid et al., 2004; Schulein et al., 2005). The Bep proteins trigger all known VirB/D4-associated phenotypic changes of infected endothelial cells (ECs), including (1) inhibition of apoptosis, (2) activation of an NF- $\kappa$ B-dependent pro-inflammatory response, (3) capillary-like sprout formation of ECs embedded in a 3D matrix, and (4) bacterial invasion of ECs as well as epithelial cells by a unique cellular structure termed the invasome (Schmid et al., 2004; Schmid et al., 2006; Rhomberg et al., 2009; Scheidegger et al., 2009; Selbach et al., 2009). Invasome formation has been shown to depend on the translocation of BepG or the combination of BepC and BepF into the recipient cells

(Rhomberg et al., 2009; Truttmann et al., 2011). *Bhe* internalization by the invasome route is a tightly controlled process, consisting of initial adherence, effector protein translocation, the formation of bacterial clusters and engulfment by plasma-membrane-derived membrane protrusions, eventually resulting in the complete internalization of the bacterial aggregates (Dehio et al., 1997; Rhomberg et al., 2009). This entry process is accompanied by the inhibition of endocytic uptake of single bacteria into *Bartonella*-containing vacuoles (BCVs) (Rhomberg et al., 2009). The small GTPases Rac1 and Cdc42, their effectors WASP and SCAR/Wave, as well as the downstream actin nucleation complex Arp2/3, have been shown to be essential for controlling F-actin rearrangements, leading to the establishment of the invasome structure (Rhomberg et al., 2009).

Several bacterial pathogens exploit the integrin family of  $\alpha\beta$  heterodimeric adhesion molecules to support internalization into host cells. For example, enteropathogenic *Yersinia enterocolitica* express the protein invasin, which binds with high affinity to the integrin  $\alpha 5\beta 1$ , thereby initiating bacteria uptake in a zipper-like manner (Isberg and Barnes, 2001). In addition to the impact of integrins in mediating bacterial entry, it has recently been demonstrated that the human pathogen *Helicobacter pylori* binds through its decorated T4SS pilus to the integrin  $\beta 1$ -subunit, resulting in the injection of the bacterial effector protein CagA

and simultaneous activation of tyrosine kinases FAK (focal adhesion kinase) and Src (Kwok et al., 2007; Jimenez-Soto et al., 2009).

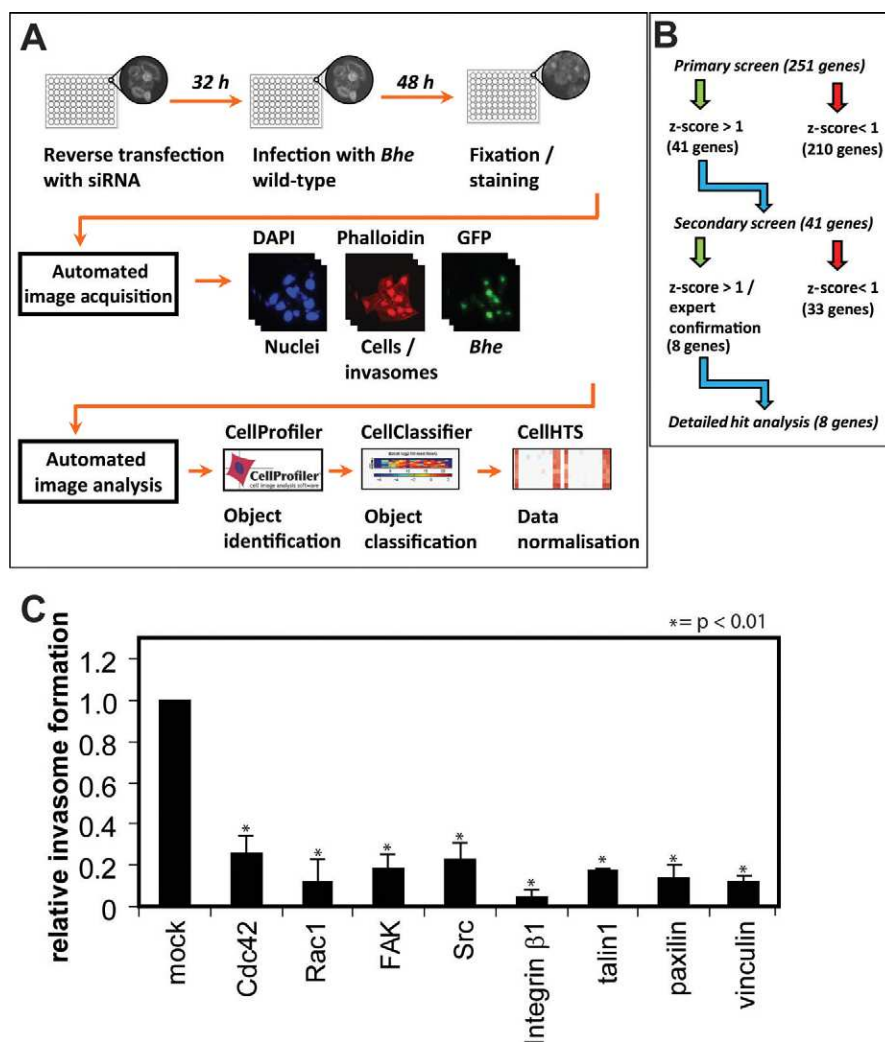
Integrins are capable of promoting bi-directional signal transduction events across the cellular membrane. Talin1-mediated inside-out activation of integrins induces a conformational change that converts integrins into an active, high affinity state (Luo and Springer, 2006; Luo et al., 2007; Dupuy and Caron, 2008; Shattil et al., 2010). Activated integrin receptors can interact with diverse extracellular ligands, thereby initiating outside-in signaling cascades that are generally associated with local remodeling of the actin cytoskeleton (Zaidel-Bar et al., 2007; Dupuy and Caron, 2008). FAK, Src kinase and adaptor proteins such as Crk, paxillin and vinculin have major roles in these events (Huvneers and Danen, 2009).

In this study, we show that *Bhe*-triggered invasome formation is dependent on integrin- $\beta$ 1-mediated signaling cascades and demonstrate that integrin- $\beta$ 1-mediated outside-in signaling mediated by FAK, Src kinase, paxillin and vinculin in combination with talin1-mediated inside-out signaling is required for invasome establishment.

## Results

### Several components associated with integrin-mediated signaling are essential for invasome formation

To investigate which host cell factors contribute to the process of wild-type *Bhe*-triggered invasome formation, we performed an immunofluorescence microscopy-based RNA interference (RNAi) screen in HeLa cells (Fig. 1A), using a spontaneous streptomycin-resistant variant of *Bartonella henselae*, ATCC49882<sup>T</sup> (Schmid et al., 2004) (referred to hereafter as *Bhe* wild-type). The short interfering RNA duplexes (siRNAs) used targeted 251 different genes (for detailed description of the experimental set-up, image analysis and hit selection see the Materials and Methods). Following data processing, 41 of the 251 targeted genes were selected for validation (supplementary material Table S1; Fig. 1B) using three additional siRNAs per gene. Out of the 41 primary hits, only 8 genes passed the more stringent hit selection of the secondary screen, in which only hits that reduced invasome formation by more than 75% compared with controls were considered for detailed investigation (Fig. 1B,C). Among these positively validated genes of the secondary screen were the small GTPases Cdc42 and Rac1, which were previously shown to contribute to invasome formation and



**Fig. 1. Schematic representation of RNAi screening set-up and results.** (A) Schematic representation of the screening set-up used in this study. (B) Flowchart summarizing the procession of hits from the primary and secondary screen as well as validated hits. (C) Quantification of wild-type *Bhe*-triggered invasome formation upon knockdown of indicated target genes ( $n > 10^3$  cells). Mock-normalized results of at least three independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from mock-transfected controls.

therefore served as internal positive controls (Rhomberg et al., 2009; Truttmann et al., 2011). Furthermore, we identified the two tyrosine kinases Src and FAK, as well as the transmembrane receptor integrin  $\beta 1$ , and the adaptor proteins paxillin, talin1 and vinculin as novel components essential for invasome formation. Interestingly, all eight proteins identified by the RNAi screen contribute to or are regulated by integrin- $\beta 1$ -mediated signaling cascades that have been shown to be essential for the internalization of other bacterial pathogens such as *Y. enterocolitica* or *Staphylococcus aureus* in an integrin- $\beta 1$ -dependent, zipper-like mechanism (Isberg and Barnes, 2001; Agerer et al., 2003).

### Integrin $\beta 1$ contributes to multiple steps in invasome formation

Because invasome formation is a multi-step process, we sought to identify at which stages the validated hits were involved. To this end, we first tested whether *Bhe* can directly interact with the transmembrane protein integrin  $\beta 1$ . Therefore, 96-well plates were coated with various matrices including recombinant  $\alpha 5\beta 1$ ,  $\alpha V\beta 3$  or  $\alpha M\beta 2$  integrins, and adhesion of wild-type *Bhe* was assessed after incubation for 60 minutes. The results showed that wild-type *Bhe* hardly adhered to surfaces coated with gelatin, fibronectin,  $\alpha V\beta 3$  or  $\alpha M\beta 2$ -integrin, whereas adherence to  $\alpha 5\beta 1$ -integrin-coated wells was significantly increased (Fig. 2A). Next, we tested for adherence of wild-type *Bhe* to the well-characterized mouse cell lines GD25, which lack integrin  $\beta 1$  (Fassler et al., 1995) and GD25  $\beta 1A$ , which overexpress integrin  $\beta 1$  (Wennerberg et al., 1996). Therefore, cells were infected for 90 minutes with *Bhe* wild-type and bacterial adherence was determined by analysis of microscopic images. The findings demonstrated that adherence of wild-type *Bhe* was significantly increased on cells expressing  $\beta 1A$  compared with the corresponding integrin- $\beta 1A$ -knockout cells (Fig. 2B).

We also investigated whether effector protein translocation was inhibited by knockdown of validated hits from the siRNA screen by performing a calmodulin-dependent adenylate cyclase (Cya) reporter assay for VirB/D4-dependent translocation (Sory and Cornelis, 1994; Schmid et al., 2006). Thus, HeLa cells were transfected with the respective siRNAs and infected with *Bhe* wild-type containing pLMS404 encoding the FLAG-Cya-BidD fusion protein, which was previously shown to be translocated into eukaryotic cells and to increase intracellular cAMP levels (Schmid et al., 2006). Wild-type *Bhe* containing pLMS400 encoding FLAG-Cya was used as a control. The results demonstrated that knockdown of integrin  $\beta 1$  led to a significant reduction of effector protein translocation, whereas knockdown of FAK, talin1, Rac1, Cdc42, Src, vinculin or paxillin had no significant effect on this process (Fig. 2C).

We then analyzed whether any of the validated hits were required for processes occurring after effector translocation. Therefore, HeLa cells were first transfected with the respective siRNAs and, following incubation for 24 hours, were transfected with a combination of plasmids pMT563 and pTR1769 encoding eGFP-BepC and eGFP-BepG effector fusions, respectively. After incubation for another 24 hours, cells were infected with the effector-free *Bhe*  $\Delta bepA-G$  mutant strain at an MOI of 500 for 48 hours, and invasome formation was quantified thereafter. Infection of GFP-BepC/BepG-expressing cells with *Bhe*  $\Delta bepA-G$  yielded approximately 8% invasome positive cells (supplementary material Fig. S1A). The results showed that knockdown of any of the validated hits led to a significant decrease in BepC/

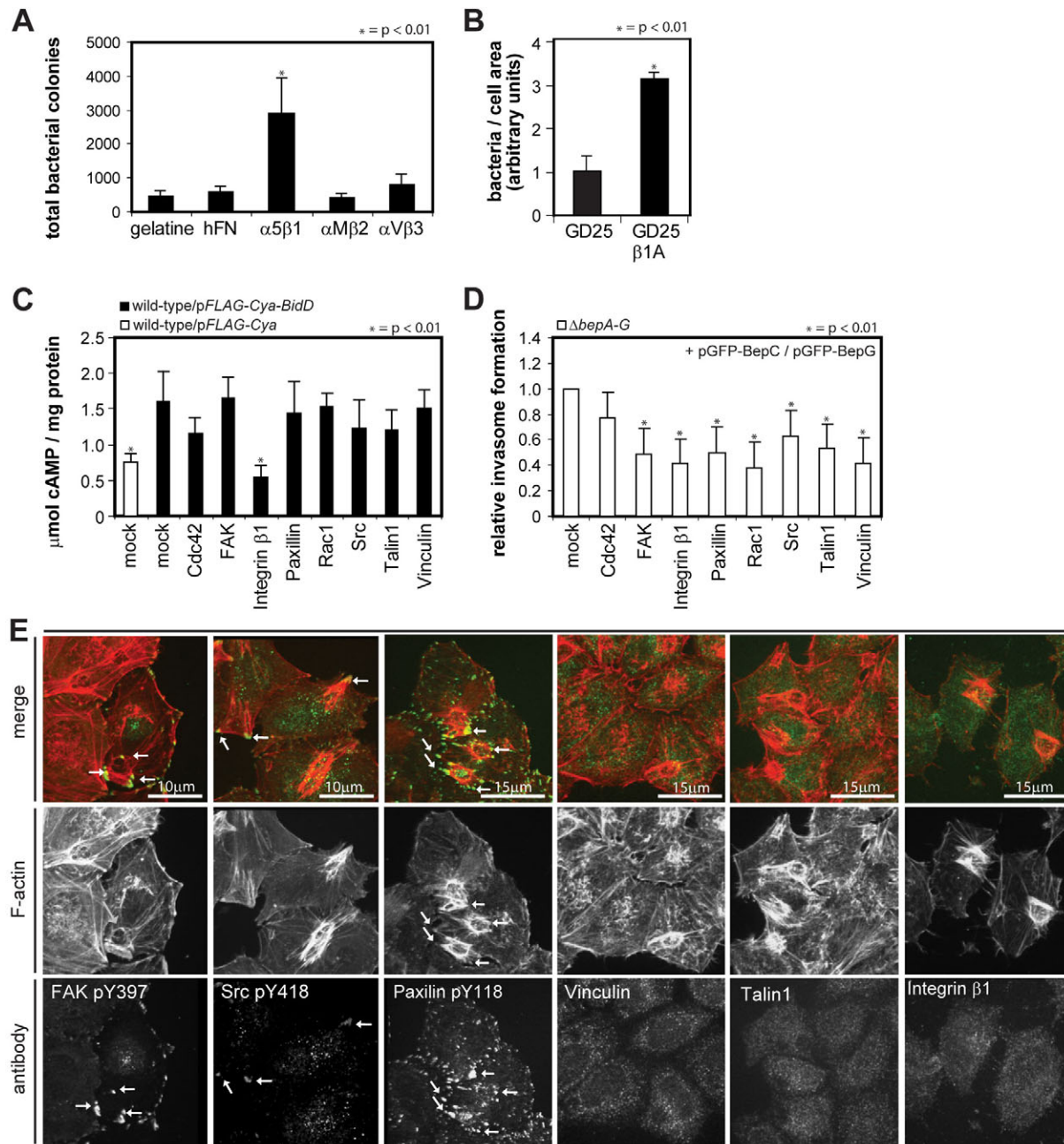
BepG-triggered invasome formation except for Cdc42 (Fig. 2D), indicating that integrin- $\beta 1$ -mediated downstream signaling is required for the F-actin recruitment process leading to the engulfment of the bacterial aggregate that eventually results in formation of invasomes.

To test for local protein enrichment of the indicated proteins with invasome structures, we stained HeLa cells infected for 48 hours with wild-type *Bhe* using different antibodies recognizing the activated forms of FAK (pY397), Src (pY418), paxillin (Y118) and integrin  $\beta 1$  (Ab12G10), as well as talin1 and vinculin (Fig. 2E). Cdc42 and Rac1 colocalization with invasome structures was previously reported (Rhomberg et al., 2009). Image analysis by confocal microscopy showed that FAK pY397, Src pY418, paxillin pY118 were all enriched at the edges of the characteristic F-actin structures. By contrast, active integrin  $\beta 1$  was only slightly enriched, and no specific enrichment of talin1 or vinculin was observed in close proximity to invasomes. Thus, the observed localization patterns indicate a local involvement of FAK, Src and paxillin in the assembly and anchoring of the F-actin cables shaping invasome structures. Taken together, our data demonstrate that integrin  $\beta 1$  binds to wild-type *Bhe* in a fibronectin-independent manner and indicate multiple roles for  $\beta 1$  integrin during the process of invasome formation.

### A BatR-regulated protein mediates interaction with integrin $\beta 1$

Recent work by other groups demonstrated that *H. pylori* binds through its T4SS directly to integrin  $\beta 1$ , thereby enabling CagA effector translocation and tyrosine phosphorylation in the host cell (Kwok et al., 2007; Jimenez-Soto et al., 2009). Because *Bhe* uses a VirB/D4 T4SS for effector translocation (Schulein and Dehio, 2002), we checked whether this apparatus could be directly binding integrin  $\beta 1$  in a BadA-independent manner. To this end, we performed adhesion- and translocation-independent invasome formation, testing different *Bhe* mutant strains. In addition to wild-type *Bhe*, we used the effector-free deletion strain *Bhe*  $\Delta bepA-G$ , the *Bhe*  $\Delta virB2-11$  mutant strain carrying a deletion of the entire *virB/D4* T4SS locus and the *Bhe*  $\Delta batR$  strain with an in-frame deletion in the transcriptional response regulatory protein BatR, which together with the sensor histidine kinase BatS, is known to form a two-component regulatory system controlling pH-regulated expression of several *Bhe* virulence genes, including the genes of the *virB/D4* locus and the adjacent *bep* effector loci (Quebatte et al., 2010). The results showed that adhesion to GD25  $\beta 1A$  cells as well as translocation-independent invasome formation was not affected by the deletion of either the *bep* effector loci or the *virB/D4* T4SS locus (Fig. 3A,B). By contrast, infections with  $\Delta batR$  led to a significant decrease in both adhesion- and translocation-independent invasome formation, thereby indicating that another bacterial factor that is neither a Bep nor part of the VirB/D4 T4SS mediates interaction with integrin  $\beta 1$  and thereby contributes to invasome formation. To test whether the interaction of wild-type *Bhe* with integrin  $\beta 1$  was mediated by recruitment of human fibronectin (FN) to the bacterial cell surface, as shown for *S. aureus* (Agerer et al., 2003), we incubated wild-type *Bhe* for 48 hours in M199 medium containing 100  $\mu g/ml$  of human FN. Immunocytochemical analysis showed that wild-type *Bhe* did not bind human FN (supplementary material Fig. S1B). Complementary analysis of invasome formation in the presence of human FN antibodies showed that depletion of human FN did not interfere with wild-type *Bhe*-triggered invasome formation

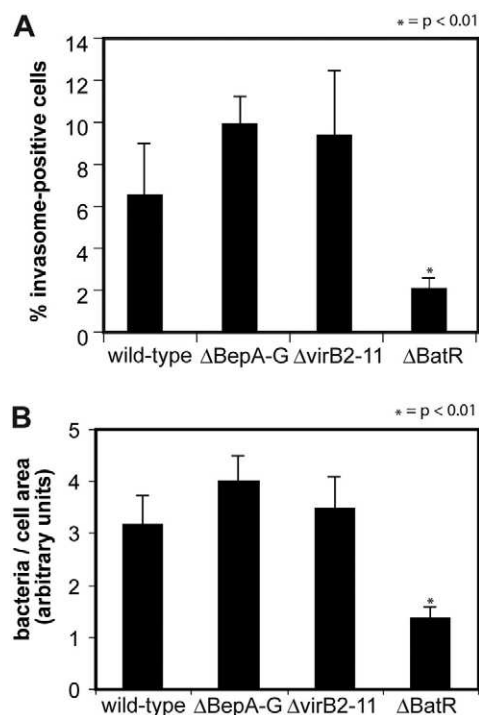




**Fig. 2. Integrin  $\beta 1$ , talin1 and FAK are engaged in multiple steps of invasome formation.** (A) Quantification of wild-type *Bhe* adherence to protein coated surfaces. Proteins used for coating are indicated. Results of three independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from gelatin-coated controls. (B) Quantification of adherence of wild-type *Bhe* to mouse fibroblasts. Specific cell lines expressing or not expressing integrin  $\beta 1A$  are indicated. Results of three independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from  $\beta 1A$ -knockout cells. (C) Analysis of *Bhe* effector translocation upon knockdown of indicated genes using Cya reporter assays. Results of three independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from controls. (D) Analysis of invasome formation on cells ectopically expressing eGFP-BepC and eGFP-BepG and infected with *Bhe*  $\Delta bapA-G$  in combination with the indicated gene knockdowns. Results of four independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from mock-transfected controls. (E) Immunocytochemical localization patterns of indicated proteins upon *Bhe* wild-type-triggered invasome formation on HeLa cells. Representative images are depicted. Colocalizing proteins are indicated with an arrow.

(supplementary material Fig. S1C). In conclusion, these results provide strong evidence that *Bhe* interacts with integrin  $\beta 1$  in a manner that is independent of VirB/D4-T4SS and fibronectin

through an unknown extracellular structure whose synthesis is transcriptionally regulated by the two-component regulatory system BatR–BatS.

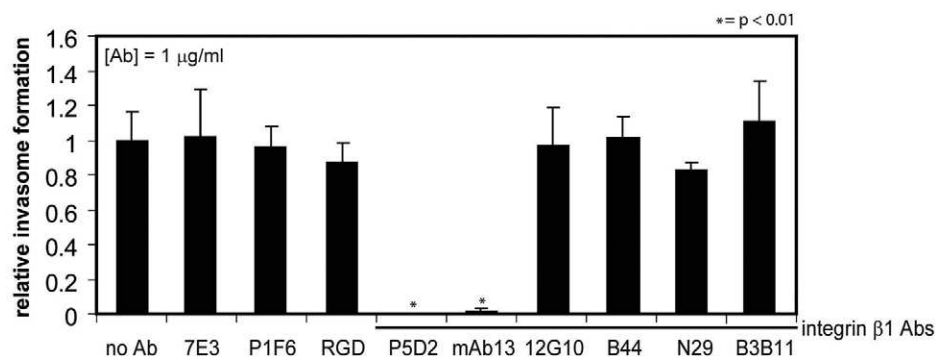


**Fig. 3. A BatR-regulated adhesin contributes to invasome formation.** (A) Quantification of *Bhe* adherence to GD25  $\beta$ 1A mouse fibroblasts. *Bhe* strains tested are indicated. Results of three independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from wild-type infected cells. (B) Analysis of invasome formation on cells ectopically expressing eGFP-BepC and eGFP-BepG and infected with indicated *Bhe* mutant strains. Results of three independent experiments  $\pm$  s.d. are depicted. Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from infected wild-type cells.

### Invasome formation requires integrin $\beta$ 1 in its extended active conformation

Integrins cycle between a bent, inactive, a primed and a fully extended active conformation (Takagi et al., 2002; Askari et al., 2009). Thus, it is expected that *Bhe* will encounter both open and closed integrins when attaching to the cells. Because our results show that knockdown of integrin  $\beta$ 1 interferes with effector protein translocation and invasome formation (Fig. 1C, Fig. 2C,D), we wanted to investigate the consequence of antibody-mediated

stabilization of the extended, or the closed conformation on invasome formation. To address this question, we infected HeLa cells with wild-type *Bhe* in the presence of different integrin  $\beta$ 1 antibody and checked for invasome formation. Antibody-promoted stabilization of either conformation prevents further cycling and will lead to the accumulation of one specific integrin conformation. A list of integrin  $\beta$ 1 antibodies used, their respective binding sites and their proposed mode of action can be found in the supplementary material Fig. S2. Inhibiting antibodies specific for integrin  $\beta$ 3 (Ab7E3) or integrin  $\alpha$ V $\beta$ 5 (AbP1F6) as well as an RGD-motif-containing peptide that binds to the A-domain of integrins served as controls. The results clearly showed that invasome formation was completely abolished in cells infected with wild-type *Bhe* and treated with the inhibitory integrin  $\beta$ 1 antibodies P5D2 or mAb13, whereas the presence of any stimulating integrin  $\beta$ 1 or control antibodies, or RGD peptide had no effect on invasome formation (Fig. 4). Next, we performed *Bhe* adhesion assays, which showed that adhesion was not affected by either stimulating or inhibiting  $\beta$ 1 integrin antibodies as well as control treatments, providing evidence that *Bhe* binds both the open as well as the closed integrin  $\beta$ 1 conformation (supplementary material Fig. S3A). To check for effector translocation in the presence of the treatments mentioned above, we performed a Cre recombinase reporter assay for translocation (CRAFT) (Schulein et al., 2005). In this assay, wild-type *Bhe* delivers a NLS-Cre-Bid fusion protein into Ea.hy926/pRS56-c#B1 recipient cells, which chromosomally encodes GFP that is transcriptionally inhibited by a *loxP*-flanked stop box. Upon NLS-Cre-Bid translocation, the fusion protein localizes into the nucleus where the Cre recombinase triggers a recombination event leading to the release of the stop box, thereby enabling GFP expression. The CRAFT results showed that inhibitory  $\beta$ 1 integrin antibodies significantly decreased effector protein delivery into the host cell whereas the other treatments did not affect effector translocation (supplementary material Fig. S3B). Furthermore, translocation-independent invasome formation assays in the presence of either stimulating or inhibitory  $\beta$ 1 integrin antibodies demonstrated that the stimulating  $\beta$ 1 integrin antibodies did not interfere with build-up of the invasome structures whereas the inhibitory  $\beta$ 1 integrin antibody completely abolished the establishment of invasomes (supplementary material Fig. S3C). In summary, our data suggest that *Bhe* is able to bind integrin  $\beta$ 1 in both the extended and the bent conformation. However, the extended integrin  $\beta$ 1 conformation is essential for allowing *Bhe* effector translocation and subsequent invasome formation.



**Fig. 4. Invasome formation in the presence of distinct integrin antibody.** Quantification of *Bhe* wild-type-triggered invasome formation in the presence of inhibiting (P5D2, mAb13) or stimulating (12G10, B44, N29, B3B11) integrin  $\beta$ 1 antibody, and inhibiting antibodies of  $\beta$ 3 integrin (7E3),  $\alpha$ V $\beta$ 5 integrin (P1F6) or an RGD-motif containing peptide ( $n > 1000$  cells). Results of at least three independent experiments  $\pm$  s.d. are depicted. Antibody concentration and antibody clone names are indicated. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from untreated controls.

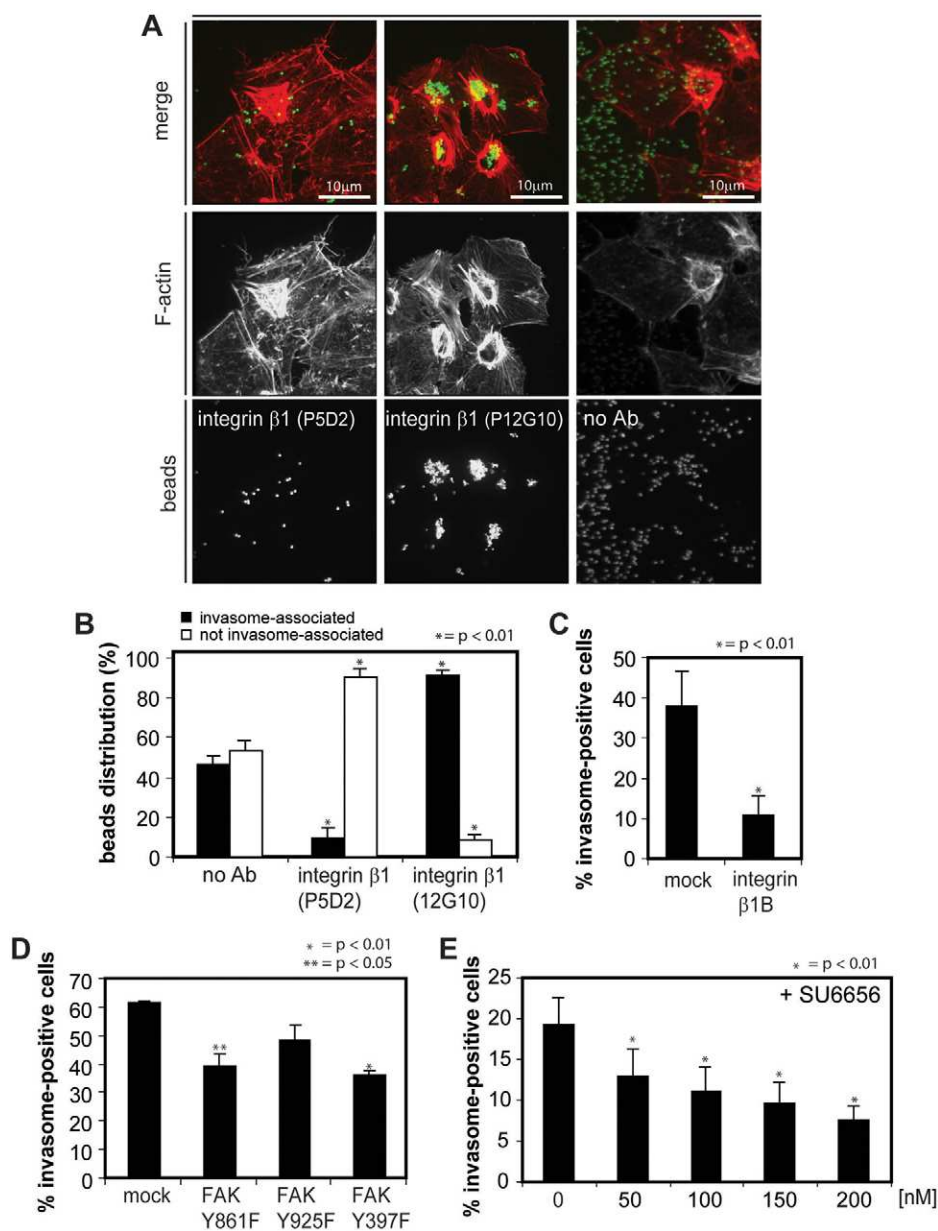
### Active integrin $\beta 1$ clusters at sites of invasome formation

The interaction of the extended integrin conformation with its ligand is often associated with integrin clustering and stimulation of integrin-mediated downstream signaling cascades (Cluzel et al., 2005; Luo and Springer, 2006). To test for a specific recruitment of active integrin  $\beta 1$  to sites of invasome formation, we first infected GFP-actin HeLa cells for 24 hours with wild-type *Bhe*. Antibody-coated microspheres were then added to the cells, and incubated for another 24 hours. The build-up of invasomes as well as the localization of the antibody-coupled microspheres was followed by time-lapse microscopy. Movies showed that microspheres coated with stimulating  $\beta 1$  integrin antibodies were recruited to sites of invasome formation and retained, whereas microspheres coated with inhibiting  $\beta 1$  integrin antibodies only transiently interacted with invasomes (supplementary material Movies 1, 2). Examination and quantification of fixed HeLa cell samples treated as described above showed that approximately 92% of microspheres coated with

stimulating integrin  $\beta 1$  antibody clustered and localized to invasomes, whereas more than 90% of microspheres coated with inhibitory integrin  $\beta 1$  antibody neither clustered, nor localized to sites of invasome formation (Fig. 5A,B). In addition, the presence of inhibitory-antibody-coated beads significantly reduced invasome formation frequency (supplementary material Fig. S4C). Taken together, these findings strongly indicate that activated integrin  $\beta 1$ , but not the inactive form, is recruited to sites of invasome formation.

### Integrin $\beta 1$ -mediated outside-in signaling via FAK and Src is required for establishment of invasomes

To date, five different integrin  $\beta 1$  isoforms have been characterized, which differ in their respective C-terminus and signaling properties. To test whether integrin- $\beta 1$ -mediated outside-in signaling contributes to invasome assembly, we transfected HeLa cells with a plasmid encoding the integrin  $\beta 1B$  isoform, which does not trigger FAK phosphorylation and reorganization of the actin



**Fig. 5. Integrin- $\beta 1$ -mediated outside-in signaling and integrin  $\beta 1$  clustering are essential for invasome formation.**

(A) Clustering of antibody-coated polystyrene beads on wild-type *Bhe* infected HeLa cells. Beads are coated with P5D2 antibody (inhibitory Ab), 12G10 antibody (stimulating Ab) or no antibody (BSA), as indicated. (B) Quantification of beads distribution on wild-type *Bhe*-infected Ea.hy926 cells. At least ten randomly chosen fields of vision were quantified per condition and experiment. Results of at least three independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from antibody-free controls. (C) Quantification of invasome formation on wild-type *Bhe*-infected cells overexpressing integrin  $\beta 1B$  in trans. Results of at least three independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from mock-transfected controls. (D) Quantification of wild-type *Bhe*-triggered invasome formation on HeLa cells expressing FAK mutants ( $n > 500$  cells); results of three independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly (\* $P < 0.01$ ; \*\* $P < 0.05$ ) from mock-transfected control wells. (E) Quantification of invasome formation triggered by wild-type *Bhe* in the presence of Src inhibitor SU6656 ( $n > 10^3$  cells). Results of three independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from DMSO-treated controls.



cytoskeleton and, moreover, displays a dominant-negative effect on the endogenously expressed forms of integrin  $\beta 1$  (Balzac et al., 1994; Armulik et al., 2000). The results showed that ectopic expression of integrin  $\beta 1B$  reduced invasome formation by approximately 70% (Fig. 5C), strongly suggesting that integrin-dependent FAK activation is crucial for invasome formation. Visual inspection of *Bhe* adhesion to HeLa cells did not show any obvious adhesion defect as a result of the overexpression of integrin  $\beta 1B$ . Next, we assessed the role of the two tyrosine kinases FAK and Src in the process of invasome formation in more detail. Therefore, HeLa cells were transfected with plasmids encoding the FAK mutants FAK Y397F, FAK Y861F or FAK Y925F before infection with wild-type *Bhe*. Tyr397 is autophosphorylated upon FAK activation, and generates a high-affinity binding site for Src kinase (Agerer et al., 2005). Residue Y861 represents the major Src phosphorylation site in FAK and is essential for the interaction of FAK with integrin tails (Lim et al., 2004). Phosphorylation of Tyr925 creates a binding site for the single SH<sub>2</sub>-domain of the adaptor protein Grb2, which links integrin activation to the Ras-MAPK pathway (Schlaepfer et al., 1994). The results showed that expression of FAK Y397F and FAK Y861F, but not FAK Y925F significantly decreased invasome formation (Fig. 5D). These findings are in line with results from the initial RNAi screen in which neither Grb2 nor any of the MAP kinases were identified as hits (supplementary material Table S1).

To validate the involvement of the Src kinase in the process of invasome formation, HeLa cells were treated with specific Src kinase inhibitors before infection. The results showed a significant dose-dependent decrease in invasome formation in response to either of two Src kinase inhibitors (Fig. 4E, supplementary material Fig. S5A). Taken together, our data suggest that outside-in signaling by integrin  $\beta 1$  through FAK and Src kinase is crucial for invasome formation. Furthermore, the Ras-MAPK pathway appears not to be involved in the process of invasome establishment.

### Talin1- but not talin2-mediated inside-out signaling by integrin $\beta 1$ is required for invasome formation

In our initial screen, we identified talin1, but not talin2, as a protein contributing to invasome formation. Talin is an adaptor protein that is essential in coupling conformational changes of integrins to downstream signaling cascades and initiates integrin activation (Tadokoro et al., 2003; Simonson et al., 2006; Anthis et al., 2009; Shattil et al., 2010). In addition, talin1 provides a link between integrins and the actin cytoskeleton (Critchley, 2009). Although talin2 is very similar (74% amino acid identity) to talin1, the role of this isoform is not yet fully understood (Debrand et al., 2009). We therefore investigated the role of both isoforms in invasome formation in more detail. Initially, we used monoclonal antibodies specific for talin1 or talin2 to show that HeLa cells express both isoforms (supplementary material Fig. S6A). Next, we tested the effects of knockdown of talin1 or talin2 on invasome formation. The siRNAs used targeted a divergent region of the two mRNAs and were shown to result in isoform-specific knockdown (supplementary material Fig. S6B,C). Infection of talin1- or talin2-knockdown cells with wild-type *Bhe* for 48 hours showed that knockdown of talin1, but not talin2 decreased *Bhe*-initiated invasome formation by more than 80% compared with mock controls (Fig. 6A). Thus, only talin1 is essential for invasome assembly.

To investigate how talin1 contributes to invasome formation, we knocked down human talin1 in HeLa cells, and then transfected the

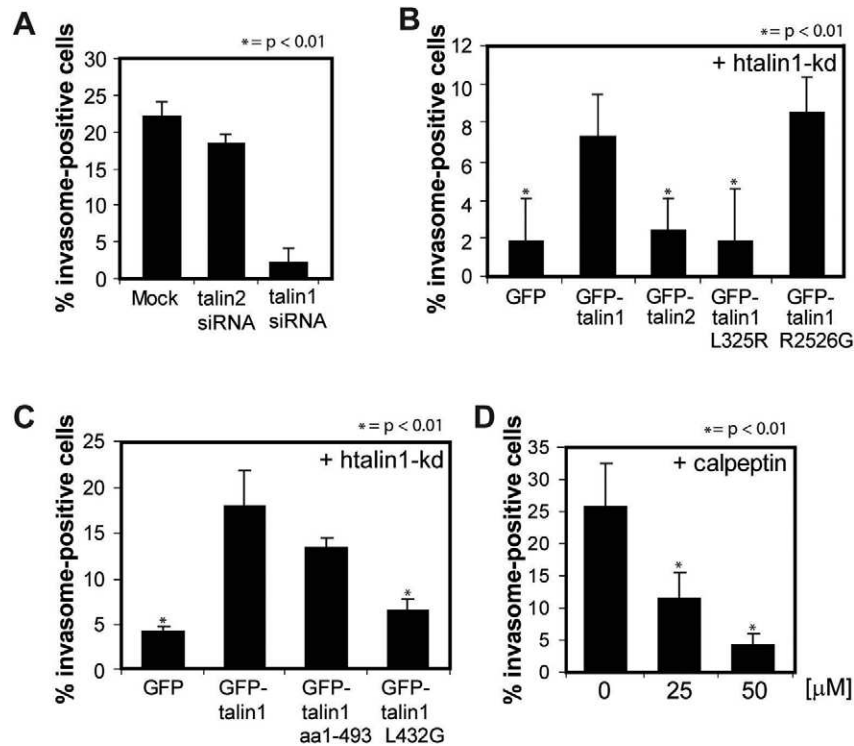
cells with plasmids encoding either wild-type GFP-tagged mouse talin1 or various talin1-GFP mutants affecting integrin activation, actin binding or calpain2 cleavage. In addition, a plasmid encoding GFP-tagged human talin2 served as control. The siRNA used to knockdown human *TALIN1* differs from the mouse *Talin1* mRNA by a single base (supplementary material Fig. S6D), and does not affect expression of mouse talin1-GFP. Expression of GFP-tagged constructs was verified by fluorescence microscopy and only GFP-positive cells were used for phenotypic quantification. Expression of wild-type mouse talin1-GFP in human talin1-knockdown cells significantly increased invasome formation compared with that in cells expressing either GFP alone or talin2-GFP (Fig. 6B). We next tested mouse talin1-GFP containing a L325R mutation in the FERM domain that compromises the ability of talin1 to activate integrins without affecting binding to the NPxY motif in  $\beta$ -integrin tails (Wegener et al., 2007). The results showed that mouse talin1-GFP L325R was unable to increase invasome formation over background levels (Fig. 6B), indicating that talin1-mediated inside-out signaling through integrins is essential to the process of invasome formation. By contrast, a GFP-tagged mouse talin1 R2526G mutation, which blocks talin1 dimerization and markedly reduces the activity of the C-terminal actin-binding site (Gingras et al., 2008; Kopp et al., 2010) was as effective as wild-type mouse talin1-GFP in supporting invasome formation. This result clearly establishes that invasome formation is not dependent on the ability of talin1 to link integrins to the actin cytoskeleton, and indicates that the main function of talin1 in invasome assembly is integrin activation. Our conclusion is supported by the finding that the isolated mouse talin1-GFP head was almost as effective as full-length talin1 in reconstituting invasome assembly in human talin1-knockdown cells (Fig. 6C).

In previous work, it has been shown that talin1 is cleaved into the head and rod domain by the Ca<sup>2+</sup>-dependent protease calpain2, and that this is required for turnover of cell-matrix junctions in cultured cells (Franco et al., 2004). Moreover, the stability of the liberated talin head is tightly regulated by the balance between cdk5-dependent phosphorylation and Smurf1-mediated ubiquitylation (Huang et al., 2009). We therefore tested the ability of a GFP-tagged mouse talin1 L432G mutant that is resistant to calpain2 cleavage (Franco et al., 2004) to support invasome assembly. Interestingly, the L432G mutant was much less effective than wild-type talin1 in contributing to invasome formation (Fig. 6C). The importance of calpain2-mediated talin cleavage in this process was further investigated by studying invasome formation in the presence of increasing concentrations of calpeptin, which is a potent inhibitor of the protease calpain2. Thus, HeLa cells were infected with wild-type *Bhe* at an MOI of 300 for 48 hours in the presence of different concentrations of calpeptin, and invasome formation was quantified in fixed and stained cells. The results showed a decrease in invasome formation of up to 80% in response to increasing concentrations of calpeptin (Fig. 6D). Together, these results suggest that liberation of the talin1 head by calpain2 cleavage contributes to invasome formation, and that the role of the talin head is to mediate inside-out activation of integrins.

### Discussion

In this study, we have investigated the host cell signaling mechanisms involved in *Bhe*-triggered invasome formation. Using a fluorescence microscopy small-scale siRNA screen, we identified six novel host cell proteins (integrin  $\beta 1$ , talin1, paxillin, vinculin, FAK, Src) that are essential for invasome formation.





**Fig. 6. Talin1- but not talin2-promoted inside-out activation of integrins contributes to invasome formation.** (A) Quantification of wild-type *Bhe*-triggered invasome formation on HeLa cells upon talin1 knockdown ( $n > 1000$  cells); results of at least three independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from mock-transfected control wells. (B) Quantification of *Bhe* wild-type-triggered invasome formation on talin1 knockdown HeLa cells expressing GFP or GFP-tagged mouse talin1, talin2, talin1 L325R or talin1 R2526G mutant in trans ( $n > 500$  cells); experiments were performed in independent triplicates; one representative is depicted here. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from GFP-talin1-expressing cells. (C) Quantification of *Bhe* wild-type-triggered invasome formation on talin1-knockdown HeLa cells expressing GFP or GFP-tagged mouse talin1, talin1 aa1-433 or talin1 L439G mutant in trans ( $n > 500$  cells); experiments were performed in independent triplicates; one representative is depicted here. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from GFP-talin1-expressing cells. (D) Quantification of *Bhe* wild-type-triggered invasome formation on HeLa cells in the presence of protease inhibitor calpeptin ( $n > 1000$  cells); calpeptin concentration are indicated. Results of at least three independent experiments  $\pm$  s.d. are depicted. Using Student's *t*-test, the data marked with an asterisk differ statistically significantly ( $P < 0.01$ ) from  $H_2O$ -treated controls.

Interestingly, all six proteins are major contributors to integrin- $\beta 1$ -mediated signaling cascades. Integrin receptors are frequently implicated in the internalization of bacteria. For example, the bacterial pathogens *Y. enterocolitica* and *S. aureus* invade host cells in integrin- $\alpha 5\beta 1$ -dependent processes (Van Nhieu and Isberg, 1991). Here, we demonstrate that the wild-type *Bhe* strain used in this study interacts in a fibronectin- and VirB/D4-T4SS-independent manner with integrin  $\beta 1$ . Previous work by Riess and colleagues (Riess et al., 2007) showed that the *Bhe* patient isolate 'Marseille' expresses a fibronectin-binding adhesin termed BadA. However, this strain lacks a functional VirB/D4 T4SS (Scheidegger et al., 2011) and therefore does not translocate the effector proteins BepC, BepF and BepG required for invasome formation (Rhombert et al., 2009; Truttmann et al., 2011). By contrast, the *Bhe* wild-type strain used in this study only encodes remnants of the *badA* gene, and although the gene is expressed, the protein is not present at the bacterial surface (Riess et al., 2007). Thus, our results suggest that another yet to be identified bacterial factor is required to mediate *Bhe*-integrin- $\beta 1$  interactions. Furthermore, our results provide strong evidence that the missing bacterial adhesin is under transcriptional control of the BatR-BatS two-component system because deletion of the regulatory protein

BatR strongly affected adhesion to cells expressing integrin  $\beta 1$ . Potential candidates include the filamentous hemagglutinins that have previously been implicated in promoting interactions between *Bordetella pertussis* and integrins (Ishibashi et al., 1994). However, the fact that *Bhe* adherence to integrin- $\beta 1$ -deficient cells was reduced but not completely abolished indicates that *Bhe* does not exclusively bind to integrin  $\beta 1$ , although this interaction is essential for invasome formation.

Our results show that integrin  $\beta 1$  is required for at least two distinct steps during invasome formation, including effector translocation. *H. pylori*, a bacterial pathogen that uses the Cag-T4SS for effector translocation but does not enter the host cell, interacts through several pili or pilus-associated proteins such as CagL, CagY or CagI directly with integrin  $\beta 1$  (Kwok et al., 2007; Jimenez-Soto et al., 2009). Jimenez-Soto and co-workers (Jimenez-Soto et al., 2009) suggested a model in which the conformational switch from the extended to the bent form of integrin  $\beta 1$  might 'pull down' the integrin-associated T4SS pilus and enable contact between the translocation system and the host cell membrane (Jimenez-Soto et al., 2009). By contrast, our results indicate that the integrin  $\beta 1$  needs to be in the extended conformation to enable *Bhe* effector translocation. The fact that

the inhibitory antibody mAb13 and the stimulating antibody 12G10 bind to the same integrin  $\beta 1$  epitope (Mould et al., 1996; Byron et al., 2010) provides strong evidence that the antibody-mediated steric hindrance is not the mechanism by which mAb13 inhibits invasome formation. Furthermore, mAb13 was shown to preferentially bind to and stabilize the bent, inactive integrin  $\beta 1$  conformation and was suggested to induce a conformational change from the open to the closed integrin  $\beta 1$  conformation in ligand-occupied, extend integrins. Our data further suggest that the capacity of bidirectional signaling of integrins is exploited during *Bhe*-promoted invasome formation. In HeLa cells, ectopic overexpression of integrin  $\beta 1B$  acting on the endogenously expressed integrin  $\beta 1$  isoform in a dominant-negative fashion or siRNA-mediated knockdown of either FAK or Src significantly decreased invasome formation, indicating a major contribution of integrin- $\beta 1$ -mediated outside-in signaling to the establishment of invasome structures. In addition, the presence of active FAK and Src at sites of invasomes support the hypothesis that locally activated signaling cascades controlled by FAK and Src that might contribute to bacteria engulfment by F-actin leading to the establishment of invasome structures. Our results also indicate that talin1-mediated inside-out activation of integrins is required for invasome formation. Interestingly, knockdown of talin1, but not talin2, reduced invasome formation by more than 80%, indicating that although talin2 is expressed in HeLa cells, it cannot compensate for loss of talin1 in this particular process. Owing to the high level of sequence identity (74%) between talin1 and talin2, it is generally assumed that the two isoforms have overlapping functions (Kopp et al., 2010). However, the calpain metalloprotease cleavage site in talin2 differs from the motif observed in talin1 and has been suggested to render talin2 resistant to proteolytic processing by calpain2 (Senetar et al., 2007). This might explain the observed specificity for talin1 in the process of invasome formation. Interestingly, human umbilical cord endothelial cells (HUVECs) that are probably the primary target cells for *Bhe* in human infections, express only talin1 and not talin2 (Kopp et al., 2010). Our finding that only talin1 is essential for invasome formation represents to the best of our knowledge the first example of a talin1-specific phenotype in a cell line expressing both talin isoforms.

The talin1 head domain binds to the C-terminal cytoplasmic domain of  $\beta 1$  integrin subunits, thereby inducing a conformational change in the integrin heterodimer from the bent, inactive to the extended, active state (Shattil et al., 2010). The importance of talin1-mediated integrin activation in invasome assembly was demonstrated by the finding that talin1-knockdown cells expressing a talin1 mutant defective in integrin activation showed a marked reduction in invasome assembly. Interestingly, invasome assembly was also compromised in talin1-knockdown cells expressing a talin1 mutant resistant to the calcium activated protease calpain2. This data plus experiments using calpain2 inhibitors suggest that the calpain2-dependent release of the talin1 head is essential for invasome formation. Indeed, the talin1 head was as effective as full-length talin1 in supporting invasome assembly in talin1-knockdown cells. In summary, we conclude that the talin1 head activates integrin  $\beta 1$  and stabilizes it in the open conformation, thereby contributing to *Bhe*-induced invasome assembly.

Based on the data presented in this study, we propose a model in which *Bhe* initially interacts with integrin  $\beta 1$ -containing heterodimeric receptors (supplementary material Fig. S7). Only bacteria bound to the extended, active integrin translocate

effector proteins and initiate early events in outside-in signaling. These initial signaling events promote a talin1-dependent inside-out activation of integrins, which we suggest increases the general level of extended integrin  $\beta 1$  on the cell surface. Thus, initial effector translocation by *Bhe* interacting with the extended integrin  $\beta 1$  conformation would prime the cells, and increase the availability of extended integrin  $\beta 1$  for subsequent interactions with *Bhe*. Clustering of *Bhe* is probably a secondary event in which the membrane proteins to which *Bhe* are bound aggregate, thereby leading to the formation of *Bhe* clusters. It is tempting to speculate that integrin  $\beta 1$  could be that receptor. The finding that beads coated with stimulating integrin  $\beta 1$  antibody are recruited to invasomes, as well as the fact that integrin activation is often linked to integrin clustering (Cluzel et al., 2005) support this hypothesis.

Our data also indicate a possible link of integrin  $\beta 1$  outside-in signaling through FAK and Src to paxillin and vinculin that might be involved in controlling the organization of F-actin leading to invasome formation. The multi-domain protein paxillin directly binds to vinculin and is involved in the regulation of small Rho-family GTPases, which in turn regulated the actin cytoskeleton (Deakin and Turner, 2008). Vinculin is a talin and actin-binding protein that stabilizes integrin-containing focal adhesions (Ziegler et al., 2006). Recently, vinculin has been demonstrated to not only bind actin but to also act as nucleation factor for de novo actin polymerization (Wen et al., 2009). Our data show that knockdown of either vinculin or paxillin abolished invasome formation almost completely. Thus, we suggest that paxillin and vinculin, together with the kinases FAK and Src locally contribute to controlling the F-actin rearrangements required to assemble invasomes. Whether or not vinculin acts as nucleation factor in this process remains unclear because published work demonstrates a crucial role for the Arp2/3 complex during invasome formation (Rhomberg et al., 2009; Truttmann et al., 2011).

Previous work on the role of talins in bacterial internalization demonstrated that the *Shigella* effector IpaA interferes with talin recruitment to integrin  $\beta 1$  tails in vitro (Demali et al., 2006). Furthermore, the injected enteropathogenic *E. coli* (EPEC) protein Tir was shown to directly bind to talin, thereby contributing to EPEC-induced pedestal formation (Cantarelli et al., 2001). In the case of *Bhe*-triggered invasome formation, we do not have any evidence yet for a direct interaction between Bep effector proteins and talin1. Nevertheless, our data clearly indicate that talin1-mediated inside-out activation of  $\beta 1$  integrins is required for invasome formation. Moreover talin1 might contribute to invasome assembly by recruiting vinculin and thereby paxillin to the membrane. However, the fact that the talin1 head domain was sufficient to rescue invasome formation indicates that the talin1–vinculin interaction might not be essential, because the vinculin binding sites are all located on the talin1 rod domain (Critchley, 2009). Nevertheless, further investigations are required to define the precise role of talin1 in invasome formation.

In summary, we demonstrate that outside-in as well as inside-out signal transduction through integrins is essential for invasome formation. In addition, we demonstrate that talin1, but not talin2 contributes to invasome formation by triggering inside-out activation of integrins. To the best of our knowledge, this is the first report that indicates active bidirectional signaling through integrins during the process of bacteria internalization in a talin1-, but not talin2-specific process.

## Materials and Methods

### Chemicals, siRNAs, antibodies, proteins and plasmids

Src inhibitors SI5 and SU6656 were purchased from Biaffin. siRNAs were purchased from Qiagen (genome-wide predesigned flexiPlate siRNAs); for all assays expect screening, only experimentally verified flexiPlate siRNAs from Qiagen were used. Functionally verified siRNAs were pooled at equimolar concentrations and used for further experiments. Antibodies and plasmids used in this study are listed in supplementary material Tables S2 and S4. Recombinant integrins were purchased from RD Systems and fibronectin from Millipore.

### Bacterial strains and growth conditions

*Bhe* ATCC49882<sup>T</sup> wild-type or isogenic mutant strains were cultured as previously described (Dehio and Meyer, 1997). For *Bhe* pre-induction, strains were cultured in M199 for 24 hours. *E. coli* strains were grown in liquid broth or solid agar plates (Luria Bertani broth) supplemented with appropriate antibiotics. Supplementary material Table S2 lists all bacterial strains used in this study.

### Construction of a *Bhe* *AvirB2-11* in-frame deletions

In-frame deletion mutants of *Bhe* RSE247 were generated as described previously by a two-step gene replacement procedure (Schmid et al., 2004) using plasmid pSH015. Plasmid description and primer sequences are provided in supplementary material Tables S2 and S3.

### Cell lines and cell culture

HeLa Kyoto  $\beta$ , HeLa Kyoto  $\beta$  GFP-actin (Snijder et al., 2009) and Ea.hy926 cells (Edgell et al., 1983) were cultured in DMEM (Gifco, invitrogen) supplemented with 10% FCS. GD25 and GD25  $\beta$ 1A (Wennerberg et al., 1996) were kindly provided by Reinhard Fässler (MPI, Munich, Germany) and were cultured in DMEM supplemented with 10% FCS.

### Generation of stable GFP-actin HeLa Kyoto $\beta$ cells

HeLa Kyoto  $\beta$ -cells were transfected with pGFP-actin (Clontech) and incubated for 24 hours in DMEM with 10% FCS. Thereafter, cells were trypsinized and suspended as 1:10–1:10,000 dilutions into 9 cm cell culture dishes containing 10 ml DMEM with 10% FCS supplemented with 20% conditioned DMEM, 10% FCS and neomycin (0.5 mg/ml). Selection pressure was kept constant for 2 weeks and GFP-positive clones were identified using a Leica DM-IRBE inverted epifluorescence microscope and thereafter transferred into 24-well plates using cloning cylinders. Upon reaching confluence in 24-well plates, individual clones were evaluated by Flow cytometric analysis using a FACSCalibur flow cytometer (Becton Dickinson) with excitation at 488 nm. Positive clones were expanded, stocked and used for further experiments.

### Infection and transfection assays

siRNA and DNA transfection and infection of HeLa cells was performed exactly as described previously (Truttmann et al., 2011). For RNAi screening, reverse transfection using pre-complexed siRNA was performed. 25  $\mu$ l of 0.5  $\mu$ M siRNA stock (Qiagen flexiplate siRNA) were mixed with 100  $\mu$ l serum-free OptiMem (Diffco, Invitrogen) in a well of a 96-well plate. Next, 125  $\mu$ l Lipofectamine 2000 (Invitrogen) was added to 12.5 ml serum-free OptiMem and cells were briefly vortexed. After 4 minutes, 125  $\mu$ l of Lipofectamine 2000 and OptiMem mix was added to each well of the 96-well plate containing the siRNA in OptiMem. The 96-well plate was sealed and put on an orbital shaker for 5 minutes. Following 25 minutes of incubation, 50  $\mu$ l of the transfection complexes mix was added into each well of an empty, dark, flat-bottom 96-well plate (Corning, Costar). Plates were sealed and stored at  $-20^{\circ}\text{C}$  until usage. On the day of experiment, plates were thawed and warmed for 60 minutes at room temperature before adding cells.

For siRNA/DNA double transfections, cells were first transfected with siRNA following standard protocols and incubated for 24 hours. Afterwards, cells were washed once with DMEM/10%FCS, transfected with DNA according to manufacturer's instructions, incubated for 6 hours, washed again, supplemented with fresh DMEM/10% FCS and eventually incubated for another 18 hours before starting with respective assays.

### Microsphere uptake and clustering assays

Microsphere uptake assays analyzing endpoint distributions have been described previously (Rhombert et al., 2009; Truttmann et al., 2011). For time-resolved microsphere clustering assays, HeLa cells expressing GFP-actin were pre-infected with the indicated strains of *Bhe* for 24 hours with an MOI of 300. Thereafter, cells were washed twice with M199 with 10% FCS and 200  $\mu$ l fresh M199 with 10% FCS containing BSA- or antibody-saturated, carboxylate-modified fluorescent (660 nm/680 nm) microspheres (0.1  $\mu$ m dark red FluoSpheres, Molecular Probes) were added to the cells (10 microspheres/cell). Plates were centrifuged for 3 minutes at 500 r.p.m. and microsphere localization and invasome formation was recorded using an MDC IXM automated microscope (Molecular Devices) equipped with an environmental control unit. Four to eight sites per well were

imaged at 10 minute intervals over 24 hours. Images were processed using MetaExpress.

### Immunoblot analysis

Protein levels were verified by analysis of total cell lysates obtained from HeLa cells. After protein separation by SDS-PAGE and transfer onto nitrocellulose, membranes were examined for the presence of the target protein specific primary antibody. Supplementary material Table S4 lists all primary antibodies used in this study. In all experiments, secondary horseradish peroxidase-conjugated antibody (Amersham, 1:10,000) was visualized by enhanced chemiluminescence (PerkinElmer).

### Immunofluorescent labeling

Indirect IF labeling was performed as described previously (Truttmann et al., 2011). Antibodies used for specific protein staining are listed in supplementary material Table S4.

### Adhesion assays

For *Bhe* adhesion assays, 96-well plates were coated overnight with 35  $\mu$ l of PBS containing 0.1% gelatin, 10  $\mu$ g/ml human fibronectin (Millipore), or 10  $\mu$ g/ml of recombinant integrin  $\alpha$ 5 $\beta$ 1,  $\alpha$ V $\beta$ 3 or  $\alpha$ M $\beta$ 2 heterodimers (RD Systems). The next day, plates were washed three times with 100  $\mu$ l PBS. Then 100  $\mu$ l of M199 containing  $10^7$  pre-induced, GFP-expressing *Bhe* were added to each well for exactly 1 hour without centrifugation. Afterwards, wells were washed three times with 100  $\mu$ l PBS and fixed with 35  $\mu$ l of 4% paraformaldehyde. Plates were imaged and *Bhe* cells were quantified using CellProfiler followed by visual inspection and manual curing where needed.

To investigate *Bhe* adhesion to mouse fibroblasts,  $4 \times 10^3$  GD25 or GD25  $\beta$ 1A cells were seeded into each well of a 96-well plate. Following overnight incubation, cells were pretreated with indicated chemicals or antibodies for at least 2 hours or left uninfected. Afterwards, cells were infected with  $10^7$  pre-induced, GFP-expressing *Bhe* in 100  $\mu$ l M199 and incubated for exactly 1 hour without centrifugation. Following washing and fixation, *Bhe* clusters were quantified using CellProfiler.

### Calmodulin-dependent adenylate cyclase reporter assay for translocation

For Cya reporter assays, 15,000 HeLa cells were seeded into wells of a 24-well plate and incubated overnight. The following day, cells were transfected and incubated for 32 hours. Thereafter, cells were infected with wild-type *Bhe* containing pMS404 or pMS400 at an MOI of 500 for 24 hours. Intracellular cAMP levels were determined using a cAMP Direct Biotrak EIA kit (GE Healthcare) according to the manufacturer's instructions and results were normalized to protein concentrations using the method of Bradford (Bradford, 1976) (Bradford Reagent, Sigma).

### Cre recombinase reporter assay for translocation (CRAFT)

CRAFT to monitor Bep translocation into the human endothelial cells has previously been described (Schulein et al., 2005). Briefly,  $2 \times 10^3$  Ea.hy926/pRS56- $\beta$ B1 cells were seeded into each well of a 96-well plate. The next day, cells were infected with wild-type *Bhe* cells containing the plasmids pRS79 at an MOI of 400 in 100  $\mu$ l M199 medium with 10% FCS and 500  $\mu$ M IPTG (Promega). Afterwards, cells were incubated for 96 hours, fixed, stained and imaged. GFP-expressing cells as well as total cell number were quantified using CellProfiler.

### Semi-automated image analysis, invasome quantification and screening hit selection

Experiments performed in 96-well plates were imaged using MDC IXM automated microscopes (Molecular Devices). 10 sites per well were imaged at  $10 \times$  magnification and each site was acquired in three wavelengths (360 nm, 488 nm, 532 nm) to visualize cell nuclei, F-actin and bacteria.

For siRNA screening experiments, invasome formation was determined by an analysis pipeline consisting of CellProfiler (Carpenter et al., 2006), Enhanced CellClassifier (Misselwitz et al., 2010) and CellHTS (Boutros et al., 2006). CellProfiler was used to identify nuclei, cells and potential invasomes, and to extract object features. For recognition of candidate invasomes, a customized CellProfiler module was developed for determining places of best fit of a ring shaped template. Details of the CellProfiler and Enhanced CellClassifier pipelines and settings are available upon request.

Primary CellProfiler output files were merged, reorganized and split using Enhanced CellClassifier (Misselwitz et al., 2010). Data points obtained from several 96-well plates ( $>10^4$  cells) were used to train SVM-Models in enhanced CellClassifier and were further used to classify candidate invasomes obtained from CellProfiler as true or false positives. Processing of each 96-well plate by Enhanced CellClassifier resulted in a list containing the percentage of invasome positive cells in a given well. These numbers of at least three independent replicates, together with well descriptions and further assay information were transferred into CellHTS (Boutros et al., 2006) to perform plate normalizations and



hit scoring (median method), where wells with a score higher than 1 were considered as potential hits. Additionally, 13 targets were picked as expert choices for validation. Secondary validation experiments were in addition analyzed semi-automatically, using MetaExpress for nuclei detection in combination with by-eye quantification of invasomes.

#### Confocal laser-scanning microscopy

Confocal laser scanning microscopy was performed as described previously (Truttmann et al., 2011).

#### Sequence alignment

Sequence alignments of siRNA and gene sequences were performed using ClustalW (Larkin et al., 2007). JalView was used for production of alignment figures (Waterhouse et al., 2009).

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